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Respiratory Infection with Attenuated *Mycobacterium tuberculosis* H37Ra in Malnourished Guinea Pigs

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Specific pathogen-free guinea pigs were infected via the respiratory route with viable, attenuated *Mycobacterium tuberculosis* H37Ra and maintained on purified, isocaloric diets. The control diet contained 30% protein (ovalbumin) and 50 ppm of added zinc (50 μg/g), the low protein diet contained 10% protein and 50 ppm of added zinc, and the low zinc diet contained 30% protein and no added zinc. Guinea pigs from each diet treatment were skin tested with purified protein derivative 48 h before sacrifice at 3, 4, and 5 weeks postinfection. Protein-deficient animals exhibited significantly reduced body weight, spleen weight, serum total proteins, and serum albumin. Zinc deficiency was characterized by loss of weight and progressive reductions in plasma zinc concentrations. The number of viable *M. tuberculosis* H37Ra cells was significantly higher in the lungs of both malnourished groups at 3 weeks, but fell below control viable counts by 5 weeks postinfection. A similar pattern was seen in the spleens and bronchotracheal lymph nodes. Both the proportion and intensity of delayed hypersensitivity reactions increased steadily between 3 and 5 weeks in control animals, whereas the two malnourished groups were essentially anergic at all intervals, despite systemic infection. These results demonstrate that both protein and zinc deficiencies exert a significant influence on the development of pulmonary tuberculosis but that the nature of the influence depends upon the interval studied. In both malnourished groups, the pulmonary infection tended to peak early and decline, whereas the disease developed more slowly in control animals. Apparent control of mycobacterial populations in the tissues was accomplished by malnourished animals in the absence of demonstrable delayed hypersensitivity.

Susceptibility to infectious diseases is altered in malnutrition (24, 40). The nature of the interaction between the nutritional status of the host and defense against microbial pathogens is complex and depends, in part, upon the nutrients and microbes involved (5). Although nutrient deficiencies, particularly protein deprivation, are usually associated with increased infectious disease morbidity, a beneficial effect of protein-calorie malnutrition has been documented in certain host-parasite relationships (9, 32, 40).

Zinc deficiency has been clearly associated with impaired humoral and cell-mediated immunity, both in humans with congenital (34) or acquired deficiencies (2) and in rodents made deficient by dietary manipulation (4, 10). Zinc is required for the function of a large number of metalloenzymes, including some involved in DNA and RNA synthesis (39). A direct effect of zinc nutriture on thymus size and mitogen-driven lymphoproliferation has been demonstrated in both humans (11, 12) and rodents (4, 10). The relevance of zinc-related immune dysfunctions for disease resistance in the deficient host has been examined in only a few studies of the response to infectious challenge (35), and there is little published evidence of such studies in guinea pigs (17).

Tuberculosis is one of the diseases which classically has been associated with malnutrition (38). A review of the early literature reveals that some studies in humans and experimental animals supported a detrimental role for malnutrition in the pathogenesis of tuberculosis (8, 36–38), whereas other investigators reported no demonstrable effect of nutritional insult on susceptibility to or severity of the disease (20, 46). Acquired resistance to mycobacteria requires an intact cell-mediated immune response (21, 23), aspects of which have been shown to be quite susceptible to modulation by nutritional manipulation (7, 45). Recent studies, both clinical and experimental, have documented the degree to which dietary deficiencies of protein and other nutrients may interfere with immunological responses to mycobacteria, especially *Mycobacterium bovis* BCG (14, 27, 29, 41). Given the importance of tuberculosis as a major cause of

morbidity and mortality in the developing world, and the potential role of nutrition in the recent field trial failure of *M. bovis* BCG vaccination in India (47), it is imperative that we understand the interaction between tuberculosis and malnutrition.

The inherent advantages of an experimental tuberculosis model employing low-level, respiratory infection of guinea pigs have been documented previously (42, 43). Using this model, Smith and colleagues (44) have delineated early events in the pathogenesis of pulmonary tuberculosis and have documented the impact of vaccination on the development of disease. In previous work, we have shown that dietary deficiencies of protein, calories, and zinc can alter immunological responses and antimycobacterial resistance in guinea pigs vaccinated with *M. bovis* BCG (29–31). We now report the influence of dietary manipulation on host responses to respiratory infection with attenuated *M. tuberculosis* H37Ra.

MATERIALS AND METHODS

Experimental animals. Outbred, albino, specific pathogen-free female guinea pigs, weighing 150 to 200 g, were obtained from a commercial source (Hartley-COBS, Crl:(HA)BR; Charles River Breeding Laboratories, Inc., Wilmington, Mass.). They were housed individually in polycarbonate cages on stainless steel mesh floors and provided food in stainless steel feeders and demineralized water (essentially zinc-free) ad libitum. Each animal was randomly assigned to an experimental diet treatment group. Body weights were recorded weekly during the experiment.

Experimental diets. The experimental diets, based upon ovalbumin as the protein source, were designed to meet current recommended nutritional requirements for guinea pigs (33). Ovalbumin is the protein source of choice for studies of dietary zinc deficiency because it contains extremely low levels of zinc contamination. The biotin content of the diet has been increased to counter the effects of avidin, a biotin-binding protein found in ovalbumin. Three diets representing combinations of an adequate level of protein (30%) with two levels of added zinc (50 and 0 ppm [50 and 0 μg/g]) and a protein-deficient (10%) diet with 50 ppm of added zinc were obtained from a commercial source (Dyets, Inc., Bethlehem, Pa.). The diets were isocaloric, with the proportion of corn starch and ovalbumin varying inversely to provide the desired protein content. The formulation of the three diets has been published previously (30). All animals were given a mixture of the fully supplemented diet (30% protein, 50 ppm of added zinc) and decreasing proportions of powdered commercial guinea pig stock diet (Ralston Purina Co., St. Louis, Mo.) over a 2-week adaptation period before assignment to one of the three experimental diets. The food was given as a powder and fresh diet was provided every other day. Food intake was monitored periodically throughout the study.

Respiratory infection. *M. tuberculosis* H37Ra (ATCC no. 25177) was obtained from the American Type Culture, Washington, D.C. The challenge inoculum was prepared and stored at −70°C according to a published procedure (13).

On the same day that the experimental diets were initiated, the guinea pigs were infected via the respiratory route in an exposure chamber essentially the same as that described by Wiegeshaus et al. (48). The concentration of viable *M. tuberculosis* H37Ra in the nebulizer fluid was adjusted empirically to result in the inhalation and retention of about 200 viable mycobacteria per guinea pig. The actual viable count was determined by plating appropriate dilutions of the challenge culture on oleic acid albumin (Difco Laboratories, Detroit, Mich.) agar plates. Groups of 18 guinea pigs, selected randomly from each of the three dietary treatments, were exposed simultaneously to the infectious aerosol. This procedure has been shown repeatedly to result in uniform, reproducible infection of all animals with mycobacteria (3, 44).

PPD skin tests. Two days before sacrifice, guinea pigs received an intradermal injection of 0.1 ml containing 100 tuberculin units of purified protein derivative (PPD-RT23; Statens Seruminstitut, Copenhagen, Denmark) on a shaved area of the side. The reactions were measured with a transparent plastic ruler 48 h later, and the mean diameter of induration was recorded in millimeters.

Autopsy procedure. Three, four, and five weeks after initiation of the experimental diets and aerosol infection, groups of six to eight guinea pigs from each treatment were killed by cervical dislocation. A blood sample (5 to 7 ml) was taken immediately from each animal by cardiac puncture into a 10-ml syringe containing sufficient preservative-free heparin (Sigma Chemical Co., St. Louis, Mo.) to provide 50 U per ml of blood. The thoracic and abdominal cavities of each animal were opened aseptically, and the right lower lobe of the lung, the bronchotracheal lymph nodes, and the spleen were removed and placed in sterile petri dishes and weighed. The tissues were then homogenized separately in 4.5 ml of sterile 2% albumin solution in a Teflon-glass homogenizer. Appropriate dilutions were inoculated onto duplicate oleic acid albumin agar plates which were incubated at 37°C for 3 to 4 weeks. The number of *M. tuberculosis* H37Ra colonies were counted, and the results were expressed as mean $\log_{10}$ viable organisms per milligram (wet weight) of tissue, to account for differences in organ size due to dietary deficiency and sampling procedures.

Hematocrits and hemoglobin concentrations were determined on the blood samples, using standard clinical procedures. Serum total proteins were quantified by the Lowry method (22), and the serum albumin concentrations were calculated by quantitative protein electrophoresis on cellulose acetate strips. Plasma zinc concentrations were measured with an atomic absorption spectrophotometer (model 303; The Perkin-Elmer Corp., Norwalk, Conn.).

Statistical Analysis. The analysis of variance was used to test the effects of the independent variables (dietary treatment, challenge to sacrifice interval) on the dependent variables (number of organisms in the tissues, skin test reaction). When appropriate, differences between group means were tested for significance with the Student's *t* test, with the probability level set at 95%.

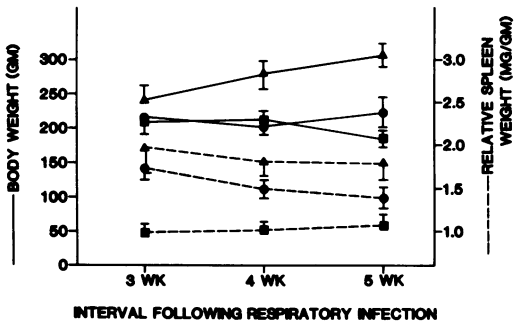

FIG. 1. Effect of diet on body weight and relative spleen weight of guinea pigs infected with *M. tuberculosis* H37Ra and maintained on control (▲), low protein (■), or low zinc (●) diets; vertical bars indicate standard error of the mean.

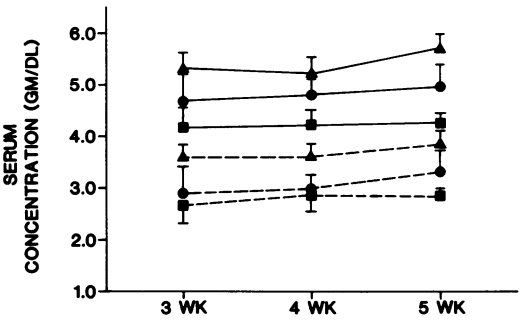

FIG. 2. Concentrations of total proteins (——) and albumin (------) in the sera of *M. tuberculosis* H37Ra-infected guinea pigs maintained on control (▲), low protein (■), or low zinc (●) diets; vertical bars indicate standard error of the mean.

RESULTS

Effect of diet on growth, protein, and zinc status. The effect of the three experimental diets on body weight and relative spleen weight of guinea pigs sacrificed at 3, 4, and 5 weeks after respiratory challenge is illustrated in Fig. 1. There was no effect on body weight at 3 weeks, but both protein- and zinc-deficient guinea pigs were significantly underweight at the 4 and 5 week intervals. The protein-deficient diet had already exerted a profound effect on spleen size by 3 weeks postinfection, whereas zinc deficiency did not result in significant splenic hypoplasia until 5 weeks. There was no significant difference in average daily food consumption between the control and low protein groups at any time. The zinc-deficient group consumed about 15 to 20% less food during the last 2 weeks of the study.

The effect of the 10% protein diet was also reflected in the serum concentrations of total proteins and albumin (Fig. 2). Levels of both were significantly reduced in protein-deficient animals at all three intervals. There was no effect of the zinc-deficient diet on serum protein concentrations, which were not significantly different from those of the fully supplemented controls.

Figure 3 documents the influence of the experimental diets on the concentration of elemental zinc in plasma. No effect was seen at 3 weeks postinfection. By 4 weeks, both protein- and zinc-deficient guinea pigs had significantly reduced levels of plasma zinc, which were diminished even further by 5 weeks in the zinc-deprived group. Plasma zinc concentrations tended to decline slightly with time even in the control group.

Time-course of *M. tuberculosis* H37Ra infection. The effect of diet on the number of viable mycobacteria recovered from the tissues differed sharply depending on the interval postinfection. In the lung (Fig. 4), both the protein- and zinc-deficient guinea pigs had significantly more organisms at 3 weeks. By 4 weeks, the number recovered from the lungs of control animals had increased, whereas the bacillary load in the two malnourished groups remained about the same. At 5 weeks postinfection, the lung viable counts had decreased in the protein- and zinc-deficient guinea pigs, while increasing still further in the control group, resulting in significantly fewer viable *M. tuberculosis* H37Ra cells in the lungs of zinc-deficient animals. Figure 5 illustrates the effect of diet and sacrifice interval on the number of viable mycobacteria recovered from the spleen. There was

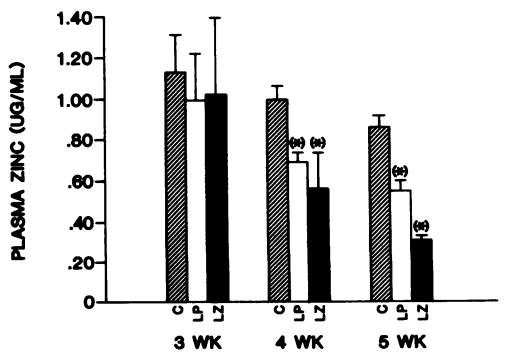

FIG. 3. Changes in plasma zinc concentrations of guinea pigs infected with *M. tuberculosis* H37Ra and maintained on control (C), low protein (LP), or low zinc (LZ) diets; mean ± standard error of the mean (vertial lines); asterisk denotes values significantly different from control ($P < 0.05$).

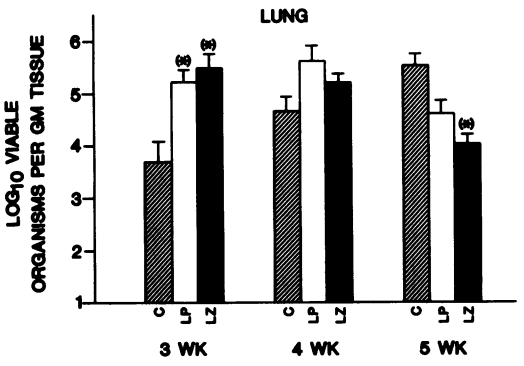

FIG. 4. Time-course of *M. tuberculosis* H37Ra infection in the lungs of guinea pigs maintained on control (C), low protein (LP), or low zinc (LZ) diets; mean ± standard error of the mean (vertical lines); asterisk denotes values significantly different from control ($P < 0.05$).

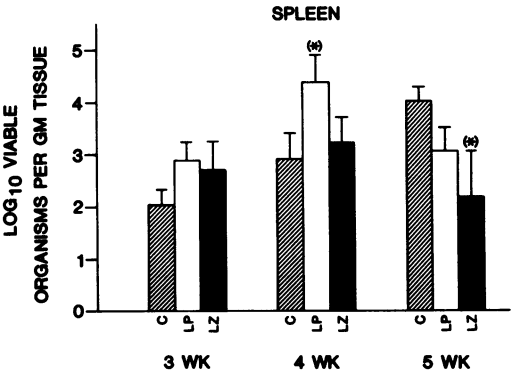

FIG. 5. Time-course of *M. tuberculosis* H37Ra infection in the spleens of guinea pigs maintained on control (C), low protein (LP), or low zinc (LZ) diets; mean ± standard error of the mean (vertical lines); asterisk denotes values significantly different from control ($P < 0.05$).

no significant effect of diet on spleen counts at 3 weeks. Significantly more organisms were recovered from the spleens of protein-deprived as compared with control animals at 4 weeks. By 5 weeks, the bacillary load had decreased in both malnourished groups, whereas viable counts from control spleens continued to rise, resulting in a significant diminution in the number of viable *M. tuberculosis* H37Ra cells in the spleens of zinc-deficient guinea pigs. A very similar picture was observed in the bronchotracheal lymph nodes (Fig. 6), where the highest bacterial counts per milligram of tissue were observed. The guinea pigs consuming a protein-deficient diet had significantly more mycobacteria in their lymph nodes at 4 weeks postinfection, whereas at 5 weeks the viable counts in control animals had increased to about the same levels observed in the two malnourished groups. Although the zinc-deficient animals had the lowest number of *M. tuberculosis* H37Ra cells in their bronchotracheal nodes at 5 weeks, the difference was not statistically significant.

Development of delayed hypersensitivity. Figure 7 illustrates the influence of diet and duration of tuberculous infection on the dermal response to PPD. In the control group, both the proportion of measurable responses and the size of the reactions increased steadily as the infection progressed. By 5 weeks, seven of nine control guinea pigs had delayed hypersensitivity reactions of 5 or more millimeters of induration. In sharp contrast, the protein-deficient animals were completely anergic at all intervals, and the zinc-deficient group contained only one responder at 3 and 4 weeks and none at the 5 week interval postinfection.

DISCUSSION

The pathogenesis of experimental pulmonary tuberculosis is altered significantly by dietary deficiencies of either protein or zinc. In guinea pigs consuming a fully supplemented diet, respiratory infection with about 10^2 viable attenuated *M. tuberculosis* H37Ra cells resulted in extensive replication of the organism in the lungs, with dissemination via the lymph and blood to other organs within 3 weeks. In control animals, the significant increase in the number of viable mycobacteria recovered between 3 and 5 weeks postinfection was accompanied by the development of delayed hypersensitivity to PPD. In

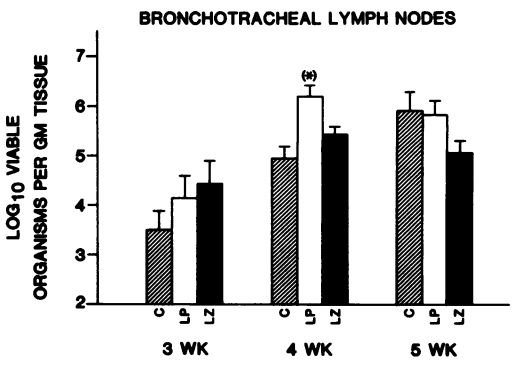

FIG. 6. Time-course of *M. tuberculosis* H37Ra infection in the bronchotracheal lymph nodes of guinea pigs maintained on control (C), low protein (LP), or low zinc (LZ) diets; mean ± standard error of the mean (vertical lines); asterisk denotes values significantly different from control ($P < 0.05$).

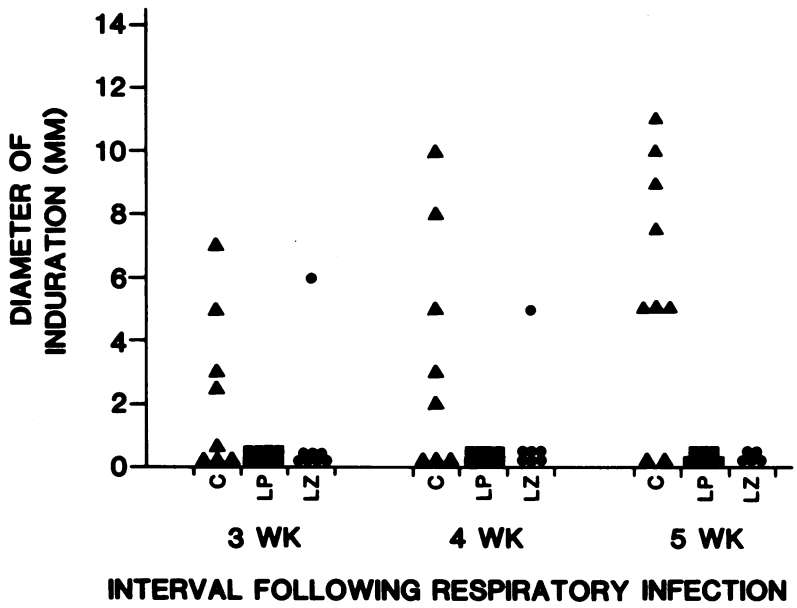

FIG. 7. Development of delayed hypersensitivity reactions to 100 tuberculin units of PPD in guinea pigs infected with *M. tuberculosis* H37Ra and maintained on control (C; ▲), protein-deficient (LP; ■), or low zinc (LZ; ●) diets.

contrast, peak levels of viable mycobacteria occurred earlier in both protein- and zinc-deficient guinea pigs and had begun to decline at 5 weeks, when viable bacterial counts in control animals were highest. Apparent control of *M. tuberculosis* H37Ra in both malnourished groups, as evidenced by stationary or declining bacterial loads in the tissues at 5 weeks, was accomplished in the absence of delayed hypersensitivity to PPD. Both protein- and zinc-deficient guinea pigs were essentially anergic at all intervals, despite systemic infection.

Previous investigators have documented the validity of bacterial enumeration in the lungs of *M. tuberculosis*-infected guinea pigs at 3 or 5 weeks postinfection as a predictor of the ultimate outcome of this host-parasite interaction (48). Using this criterion in the present study, one would reach a very different conclusion depending upon the interval examined. At 3 weeks, the malnourished animals were clearly at a disadvantage, with significantly more mycobacteria in their tissues. In sharp contrast, the data at 5 weeks support just the opposite conclusion, i.e., that the zinc-deficient guinea pigs were controlling their bacterial antagonist more successfully than were the normally nourished animals.

The metabolic responses of the guinea pigs to consumption of the experimental diets for 3 to 5 weeks were similar to those observed in previous work with this model (28, 30, 31). The 10% ovalbumin diet resulted in a significant degree of protein malnutrition, as measured by decreased body weight, hypertrophy of lymphoid organs, and a marked diminution in serum protein concentration. This effect appears to be mediated entirely by protein deprivation, since we observed no differences in food consumption between the 30 and 10% protein groups. Zinc deficiency in guinea pigs consuming a diet containing adequate protein (30%) but less than 5 ppm of elemental zinc was characterized by modest growth retardation due primarily to a 15 to 20% reduction in food consumption and by a progressive and significant reduction in plasma zinc concentrations. Zinc levels were also reduced in the protein-deficient group at 4 and 5 weeks, although not to the extent of the zinc-deprived animals (Fig. 3). This phenomenon has been observed before by us and others (30; S. M. Filteau and B. Woodward, Am. J. Clin. Nutr. 35:xvii, 1982) and may be due to the effect of protein-deficiency on zinc-binding ligands in the intestinal mucosa or peripheral circulation, which could alter zinc absorption, mobilization, or both. The potential influence of this "secondary" zinc deficiency in the protein-deprived animals on immune function and disease resistance should not be ignored. Plasma zinc concentrations in control guinea pigs were somewhat lower than normal values reported previously (1, 25) and tended to decline between 3 and 5 weeks postinfection. Depressed plasma zinc levels,

which we have observed also in well-nourished *M. bovis* BCG-infected guinea pigs (30), may be due to redistribution of zinc with uptake by the liver (6). It is conceivable that the experimental diets affected the availability of other micronutrients (e.g., iron or magnesium) in the malnourished guinea pigs, but there is little evidence to support that hypothesis.

The apparently contradictory nature of our observations mirrors the dichotomy of conclusions reached by previous investigators who have examined the interactions among malnutrition, immunity, and infectious disease. Although we and others have found that delayed hypersensitivity responses are nearly always profoundly suppressed in malnutrition (5, 7, 14, 27, 29, 41, 45), other aspects of cell-mediated immunity may be enhanced, including macrophage-mediated phenomena (17, 19, 25, 31). The latter observations may have particular relevance for host resistance against facultative intracellular pathogens such as the mycobacteria. Indeed, suboptimal dietary intake of certain nutrients can actually tip the host-parasite balance in favor of the host (9, 32, 49).

It may be trite to state that the outcome of such studies depends upon the nature of the model system employed, including the virulence of the microorganism, the route and dose of infection, the measures used to assess antimicrobial resistance, and the time at which the measurements are made. For example, the influence of protein or zinc nutriture on mycobacterial populations in the lungs of *M. tuberculosis* H37Ra-infected guinea pigs after respiratory challenge is distinct from the effect of identical diets on levels of *M. bovis* BCG in the inguinal lymph nodes of animals vaccinated subcutaneously (30). Jakab and colleagues (15) have demonstrated clearly the influence of the type of challenge organism on the alteration of murine lung defense mechanisms by dietary protein depletion. In this study, the use of an attenuated strain of *M. tuberculosis* may not have provided a rigorous test of the antimicrobial defenses of the malnourished animals. Previous investigators have shown that whereas *M. tuberculosis* H37Ra can replicate and become disseminated to extrapulmonary sites in guinea pigs, it produces a self-limiting disease (3). Ratcliffe and Merrick found that protein-malnourished animals were impaired in their ability to control virulent *M. tuberculosis* H37Rv after respiratory infection (36, 37), suggesting that our results might have been different if virulent mycobacteria were employed.

Tuberculin hypersensitivity is usually related to acquired resistance to *M. tuberculosis* in this guinea pig model (18). There are a number of circumstances, however, in which dermal reactivity to PPD is diminished or absent even in the presence of a vigorous, protective immunity (26). Delayed hypersensitivity reactions are sensitive to nutritional insult (7, 14, 27, 29, 30, 41, 45). In both guinea pigs and humans, statistical correlations have been established between protein status, as measured by serum albumin and hemoglobin concentrations, and the size of dermal responses to PPD at 48 h (16, 31). We and others have demonstrated that malnourished animals are apparently capable of controlling the accumulation of intracellular bacterial pathogens such as *M. bovis* BCG (29) and *Listeria monocytogenes* (49) in the absence of demonstrable delayed hypersensitivity. A similar conclusion can be drawn from the results presented here. Taken together, these observations suggest that this peripheral manifestation of cell-mediated immunity is not a valid indicator of the immune (i.e., protected) status of the malnourished host. This may reflect differential sensitivity of effector and regulator lymphocyte subsets to nutritional stress or the presence of humoral immunoregulatory substances which suppress dermal reactivity without affecting internal antimicrobial defenses (16). Alternatively, malnourished individuals may rely upon mechanisms for activating macrophages which do not depend upon classical T-lymphocyte mediators or other cytotoxic strategies such as natural killer cells or antibody-dependent cellular cytotoxicity. Both natural killer cells and antibody-dependent cellular cytotoxicity have been found to be enhanced in zinc deficiency (17, 25).

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